

REMARKS

In response to the Office communication mailed June 9, 2004, Applicant has amended the specification and claims to provide the chemical name of the elected XX5, namely Ro-1724 ((4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone)(Ro-20-1724)).

This is clear from page 7, lines 2-9, and the references cited there. Specifically, page 7 describes two Type 4 enzyme inhibitors, referred to as rolipram and "the structurally related compound XX5." The specification immediately cites two references Schwabe et al. and Sheppard et al. (copies attached hereto).

Schwabe et al., published in 1976, discloses rolipram ((4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone) (ZK 62711)). In this publication, Schwabe compares rolipram with the structurally related compound Ro-1724 and teaches that rolipram, which is closely related in structure to Ro 20-1724, is more potent than Ro-1724 (i.e., discussion on page 908).

Sheppard et al., published in 1972, also discusses Ro-1724 and teaches in that article that Ro-1724 was the preferred PDE4 inhibitor.

Thus, it is evident to those of skill in the art that XX5 is referring to ((4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone), also referred to as Ro-1724.

In view of the foregoing, Applicant respectfully submits that all claims are in condition for allowance. Early and favorable action is requested.

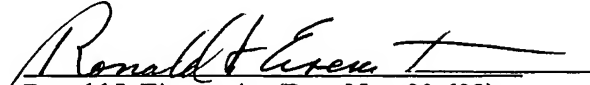
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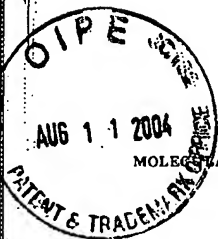
Application No. 10/060,759
Office Communication dated June 9, 2004
Response to Office Communication mailed August 9, 2004

In the event that any additional fees are required, the PTO is authorized to charge our deposit
account No. 50-0850.

Respectfully submitted,

Date: August 9, 2004


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MOLECULAR PHARMACOLOGY, 12, 900-910

4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a Potent Inhibitor of Adenosine Cyclic 3',5'-Monophosphate Phosphodiesterases in Homogenates and Tissue Slices from Rat Brain

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(Received April 26, 1976)

(Accepted June 28, 1976)

SUMMARY

SCHWABE, U., MIYAKE, M., OHGA, Y. & DALY, J. W. (1976) 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3',5'-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. *Mol. Pharmacol.*, 12, 900-910.

A new class of inhibitor of phosphodiesterases is represented by 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711). This compound enhances the magnitude of accumulations of cyclic 3',5'-AMP elicited by norepinephrine, isoproterenol, histamine, and adenosine in rat cerebral cortical slices and by veratridine in rat cerebellar slices and, in addition, increases basal levels of cyclic AMP in both cortical and cerebellar slices. Increases in intracellular levels of cyclic AMP in brain slices elicited by ZK 62711 do not appear to involve enhanced "release" of adenosine, since both basal and norepinephrine-elicited accumulations of cyclic AMP are increased by ZK 62711 in the presence of exogenous adenosine deaminase. ZK 62711 has little effect on levels of cyclic GMP in cortical or cerebellar slices. In rat cerebral homogenates ZK 62711 inhibits soluble and particulate cyclic AMP phosphodiesterases but is less potent with respect to cyclic GMP phosphodiesterases. At low concentrations it is 100 times more potent than a structurally related phosphodiesterase inhibitor, Ro 20-1724, with respect to the calcium-dependent cyclic AMP phosphodiesterase, and in brain slices it is similarly 100 times more potent in enhancing accumulations of cyclic AMP elicited by norepinephrine.

INTRODUCTION

Phosphodiesterase inhibitors have proven useful tools for the elucidation of the role of cyclic nucleotides in the control of cellular physiology in a variety of tissues and cell types. However, in brain tis-

sue the usefulness of the established phosphodiesterase inhibitors is severely limited because of side effects. These phosphodiesterase inhibitors include theophylline, 3-isobutyl-1-methylxanthine, papaverine, dipyridamole, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), diazepam, phenothiazines, and 1-ethyl-4-isopropylidene hydrazino-14-pyrazolo(3,4)pyridine-5-carboxylate ethyl ester (SQ 20,009) (1-5). Since adenosine appears to play a unique and pivotal role in the control of

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done (ZK 62711): a
Monophosphate
Slices from Rat Brain

J. W. DALY

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6) 4-(3-Cyclopentyloxy-4-
methylphenoxy)-2-pyrrolidinone
and tissue slices from rat

d by 4-(3-cyclopentyloxy-4-
methoxyphenyl)-2-pyrrolidinone
enhances the magnitude of
the response to isoproterenol, histamine,
and norepinephrine in rat cerebellar slices
from both cortical and cerebellar
slices elicited by ZK 62711
since both basal and norepi-
dine elicited by ZK 62711 in the pres-
ence of theophylline. The effect on levels of cyclic
AMP generated by ZK 62711 inhibits
adenosine uptake is less potent with respect to
theophylline, 10 times more potent than a
1:100, with respect to the cal-
cium-dependent cyclic AMP elicited by norepineph-

ness of the established phos-
phodiesterase inhibitors is severely lim-
ited. These phosphodiesterase inhibitors
include theophylline, isobutylmethylxanthine,
papaverine, 4-(3-butoxy-4-methoxyben-
zyl)-2-imidazolidinone (Ro 20-1724), diaze-
pam, and 1-ethyl-4-isopropyl-10-pyrazolo(3,4-d)
pyridine ethyl ester (SQ 20,009).
Levosimvastatin appears to play a
vital role in the control of

cyclic 3',5'-AMP-generating systems in
brain tissue (6, 7), any phosphodiesterase
inhibitor which inhibits or augments
adenosine mechanisms possesses limited
usefulness in delineating the role of phos-
phodiesterases in intact brain cells. Theo-
phylline and isobutylmethylxanthine are
potent adenosine antagonists (8, 9). Other
phosphodiesterase inhibitors, such as pa-
paverine and dipyridamole, are potent in-
hibitors of uptake of adenosine and thus
prevent reuptake of the endogenous adeno-
sine which is continually released from
brain slices (10). Much of the effect of these
compounds on cyclic AMP levels is due not
to inhibition of phosphodiesterase, but
rather to enhanced activation of adeno-
sine-sensitive cyclases. Ro 20-1724 has
been found to inhibit uptake of adenosine
into brain slices (8) and appears to have a
strong adenosine component in its poten-
tiative effects on biogenic amine-elicited
accumulations of cyclic AMP in brain
slices (8, 11-13). In addition, Ro 20-1724
has activity as a monoamine oxidase in-
hibitor (14). Diazepam inhibits uptake of
adenosine into brain slices (8), and thus
the effects of this component on cyclic
AMP levels in brain slices must be consid-
ered (8, 15). Phenothiazines, such as chlor-
promazine and trifluoperazine, are moder-
ately active phosphodiesterase inhibitors
with rather selective effects on the cal-
cium-dependent brain phosphodiesterases
(16). In brain slice preparations, phenothi-
azines usually inhibit rather than aug-
ment accumulations of cyclic AMP, proba-
bly through inhibition of adenylate cy-
clases (17-19). SQ 20,009, a very potent
inhibitor of brain phosphodiesterases, has
little or no effect on accumulations of cyclic
AMP in brain slices (8, 11, 12), although 1
mM SQ 20,009 has been reported to en-
hance dopamine-elicited accumulation of
cyclic AMP in rat striatal slices (20). SQ
20,009 inhibits uptake of adenosine into
brain slices (8). It is readily apparent that
no selective and potent inhibitor of phos-
phodiesterases in brain tissue is available.
It is therefore of interest to investigate
other compounds as inhibitors of phos-
phodiesterases in brain slices and cell-free
preparations. A centrally active 4-
(phenyl)-2-pyrrolidinone (ZK 62711, Fig.

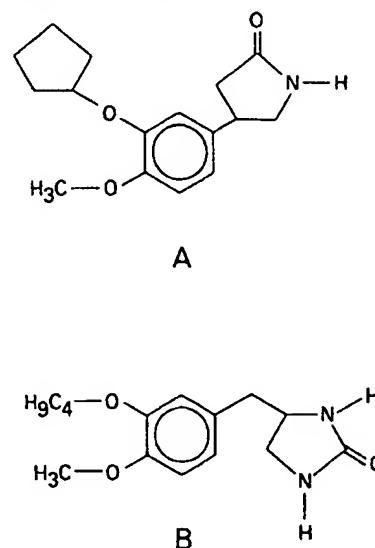


FIG. 1. Structures of 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone (ZK 62711) (A) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) (B)

1A) has been found to potentiate prosta-
glandin E_1 -elicited accumulations of cyclic
AMP in thrombocytes at low concentra-
tions.³ In view of the potency of ZK 62711
in thrombocytes, its structural resem-
blance to the 4-(benzyl)-2-imidazolidinone,
Ro 20-1724 (Fig. 1B), and the central de-
pressant activity of both ZK 62711 and RO
20-1724, an investigation of the effects of
this pyrrolidinone on cyclic AMP levels in
brain slices was undertaken. ZK 62711 was
found to be a potent agent with respect to
enhancement of amine- or adenosine-elic-
ited accumulations of cyclic AMP in rat
brain slices and with respect to inhibition
of calcium-dependent cyclic AMP phos-
phodiesterases from rat brain.

MATERIALS AND METHODS

Tissue preparation. Male Sprague-Daw-
ley rats (180-220 g) were decapitated, and
their brains were removed and chilled in
ice-cold Krebs-Ringer-bicarbonate-glucose
(21). Longitudinal strips of cerebral cortical
gray matter or pieces of cerebellar gray
matter from two or three rats were
chopped on a McIlwain tissue chopper set
at 260 μ m. The cerebral cortical slices

³ W. Kehr, personal communication.

were incubated for 40 min in 20 ml of Krebs-Ringer-bicarbonate-glucose gassed with 95% O₂-5% CO₂ throughout all incubations. The slices then were washed, collected on fine nylon mesh, and divided into portions which were transferred into 12-22 incubation beakers for an additional incubation period of 30 min in order to reduce the cyclic AMP content to a constant low basal level (13). Each beaker contained 20-40 mg of tissue per 10 ml of medium. Cerebellar slices were incubated for 40 min, divided, transferred to 12-22 separate beakers, and incubated for an additional 60 min. Phosphodiesterase inhibitors were added 2 min prior to addition of other agents (see legends to figures and tables). The final incubation with agents was carried out for 10 min. The incubations were terminated by collection of slices on fine nylon mesh and rapid transfer to ground glass homogenizers containing 1 ml of 5% trichloroacetic acid, followed by immediate homogenization and centrifugation. Cyclic AMP and cyclic GMP were measured in trichloroacetic acid supernatants after addition of 1 N HCl, extraction with ether, and lyophilization (13). Cyclic AMP was measured by the protein binding method of Gilman (22). Cyclic GMP was determined by the radioimmunoassay of Steiner *et al.* (23), using a Schwarz/Mann assay kit. Further purification of the cyclic AMP or cyclic GMP by column chromatography did not affect the results. Previous experiments had demonstrated virtually complete reduction of cyclic AMP and cyclic GMP levels by incubation of supernatants with phosphodiesterases. The trichloroacetic acid precipitates were solubilized with 1 N NaOH and assayed for protein according to the method of Lowry *et al.* (24) as modified by Miller (25).

Preparation of rat brain phosphodiesterases. Soluble and particulate phosphodiesterases of rat brain were prepared according to the methods of Russell *et al.* (26). Tissue from rat cerebrum was homogenized in 9 volumes of 0.32 M sucrose, 5 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 0.1 mM dithiothreitol with a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min, and

the supernatant solution was dialyzed overnight against 20 mM Tris-HCl, pH 7.5, containing 1 mM MgCl₂ and 0.1 mM dithiothreitol. Aliquots of the dialyzed solution were used as the source of soluble phosphodiesterases. The pellet was resuspended in sucrose solution equal to the original volume of homogenate, and resuspended by homogenization. After a second 100,000 × *g* centrifugation, the pellet was resuspended in sucrose solution. This washed particulate preparation was solubilized by sonication with a Branson Sonifier (50 W, 30 sec/ml), and the residue was removed by centrifugation at 30,000 × *g* for 30 min. The solubilized solution was dialyzed as described above. Aliquots of the dialysate were used as the source of particulate phosphodiesterases. Calcium-dependent activator protein for phosphodiesterase was prepared from rat cerebrum through heat treatment, DEAE-cellulose chromatography, and Sephadex G-75 gel filtration according to Lin *et al.* (27), with slight modifications.

Phosphodiesterase assays. The enzyme activity was measured by the procedure of Boudreau and Drummond (28). An appropriate dilution of enzyme (1.3-5.6 µg of protein per tube) was incubated in 50 mM Tris-HCl buffer (pH 8.0) containing 3 mM MgCl₂, 0.1 mM dithiothreitol, 1 µM cyclic AMP or cyclic GMP, and 2 × 10⁵ cpm of cyclic [³H]AMP or cyclic [³H]GMP. Either 0.5 mM EGTA⁴ or 0.04 mM CaCl₂ plus 1 µg of activator protein was present. The total volume was 0.5 ml. Following incubation for 20 min at 30°, the reaction was terminated by placing the tubes in a Dry Ice-acetone bath. The tubes were then placed in a boiling water bath for 3 min, followed by chilling in ice. The solutions were then incubated with 0.1 ml of a snake venom solution (1 mg/ml) for 20 min at 30°. The reaction was terminated by the addition of 1 ml of AG1-X2 ion-exchange resin (1:3 slurry of resin in H₂O containing 15 mM acetic acid for the cyclic AMP phosphodiesterase assay, or in 80 mM formic acid for the cyclic GMP phosphodiesterase assay).

⁴ The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

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it solution was dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂ and 0.1 mM dithiothreitol. The source of soluble phosphodiesterase was a pellet that was resuspended in an equal volume of the original volu-
nate, and resuspended by . After a second 100,000 × g centrifugation, the pellet was resuspended in the same solution. This washed preparation was solubilized by a Branson Sonifier (50 W, 10 sec), the residue was removed by centrifugation at 30,000 × g for 30 min. The supernatant solution was dialyzed as above. Aliquots of the dialysate were used as the source of particulate phosphodiesterase. Calcium-dependent phosphodiesterase was purified from rat cerebrum through DEAE-cellulose chromatography on Sephadex G-75 gel filtration *in et al.* (27), with slight

modifications. The enzyme was assayed by the procedure of Drummond (28). An aliquot of enzyme (1.3–5.6 μg of protein) was incubated in 50 mM Tris-HCl (pH 8.0) containing 3 mM dithiothreitol, 1 μM cyclic GMP, and 2 × 10⁵ cpm of [³H]cyclic GMP. Either 0.04 mM CaCl₂ plus 1 μg of protein was present. The total volume was 1 ml. Following incubation at 30°C, the reaction was terminated by placing the tubes in a Dry Ice-ethanol bath for 3 min, followed by centrifugation. The solutions were then extracted with 0.1 ml of a snake venom (1:3 in H₂O containing 15 mM Tris-HCl, pH 8.0, 15 mM NaCl, 15 mM cyclic AMP phosphodiesterase in 80 mM formic acid for phosphodiesterase assay).

Other reagents used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

After centrifugation, 0.5 ml of supernatant solution was counted in 10 ml of scintillation fluid.

Materials. Adenosine, *l*-norepinephrine HCl, *l*-isoproterenol bitartrate, dopamine HCl, adenosine cyclic 3',5'-monophosphate, guanosine cyclic 3',5'-monophosphate, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid, adenosine deaminase, and snake venom (*Ophiophagus hannah*) were purchased from Sigma Chemical Company. The adenosine deaminase was diluted 10-fold with a 1% solution of bovine serum albumin the day before use and dialyzed overnight against 100 volumes of distilled water to remove the high content of ammonium sulfate. Histamine 2HCl and veratridine were purchased from Aldrich Chemical Company. Anion-exchange resin AG1-X2, chloride form (200–400 mesh), was purchased from Bio-Rad Laboratories. 4-(3-Cyclopentyl-4-methoxyphenyl)-2-pyrrolidone (ZK 62711) was kindly provided by Dr. Wolfgang Kehr of Schering, Berlin, and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), by Dr. W. E. Scott of Hoffmann-La Roche. [³H]Adenosine 3',5'-monophosphate (specific activity, 27 Ci/mmol) and [³H]guanosine 3',5'-

monophosphate (specific activity, 19 Ci/mmol) were purchased from Amersham/Searle.

RESULTS

The effect of ZK 62711 on basal levels of cyclic AMP and on accumulations of cyclic AMP elicited by norepinephrine and adenosine were investigated in rat cerebral cortical slices (Fig. 2). ZK 62711 elevated basal levels of cyclic AMP by about 4–5-fold, with a maximal effect at approximately 30 μM concentration. ZK 62711 enhanced the responses to maximal stimulatory concentrations of norepinephrine and adenosine approximately 2-fold, with an EC₅₀ of about 0.4 μM for the norepinephrine response and about 10 μM for the adenosine response. In view of the structural resemblance of ZK 62711 and Ro 20-1724, the two compounds were compared with regard to effects on norepinephrine-elicited accumulations of cyclic AMP in rat cerebral cortical slices (Fig. 3). Both compounds enhanced the response to maximal stimulatory concentrations of norepinephrine by about 2-fold, but ZK 62711 was nearly 100 times more potent than Ro 20-1724.

The effect of Ro 20-1724 on accumula-

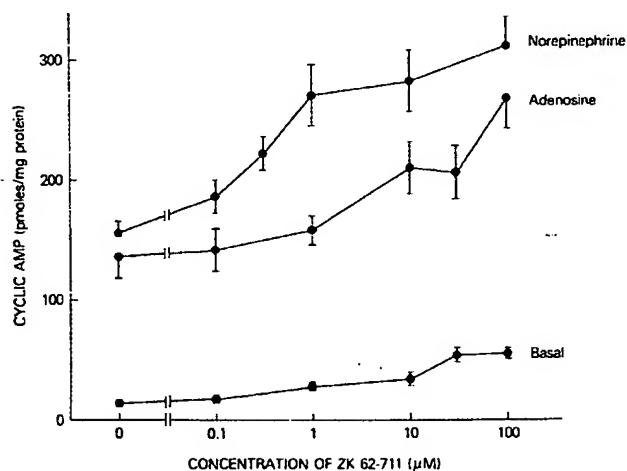


FIG. 2. Effect of ZK 62711 on accumulations of cyclic AMP elicited by norepinephrine and adenosine in rat cerebral cortical slices.

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Various concentrations of ZK 62711 were added 2 min prior to 100 μM norepinephrine or 100 μM adenosine. Final incubations were then carried out for 10 min. Values represent means ± standard errors for four separate experiments.

tions of cyclic AMP in brain slices has been shown to involve not only inhibition of phosphodiesterase, but also enhanced activation of cyclases by endogenous adenosine (8). In order to investigate possible involvement of endogenous adenosine in the potentiative effect of ZK 62711 on responses of cyclic AMP-generating systems to norepinephrine in brain slices, studies were carried out in the presence of adenosine deaminase. Adenosine deaminase

eliminates contributions of adenosine "released" from cells into extracellular space by converting adenosine to the inactive metabolite inosine (8, 29). The presence of adenosine deaminase largely prevents the effect of Ro 20-1724 on levels of cyclic AMP (results not shown). In the presence of adenosine deaminase ZK 62711 still increased basal levels of cyclic AMP by about 4-fold (Fig. 4), indicating that, in contrast to various other phosphodiester-

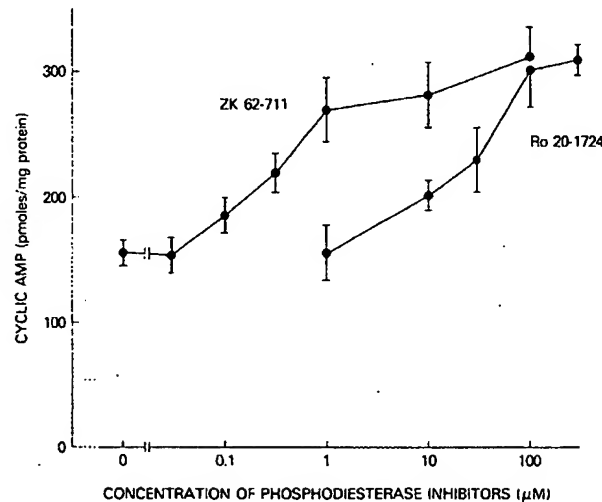


FIG. 3. Accumulation of cyclic AMP elicited by norepinephrine in rat cerebral cortical slices: potentiation by ZK 62711 and Ro 20-1724

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Either ZK 62711 or Ro 20-1724 at various concentrations was added 2 min prior to 100 μM norepinephrine. Final incubations were then carried out for 10 min. Values represent means \pm standard errors for four or five separate experiments.

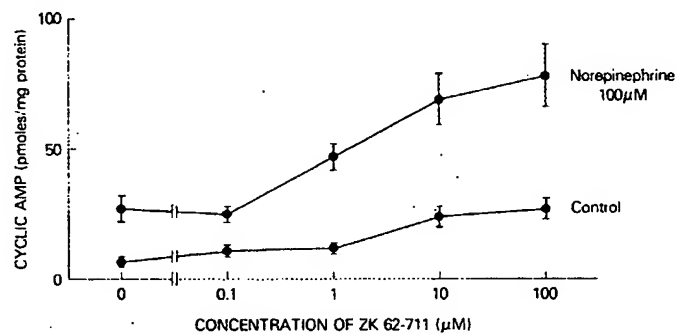


FIG. 4. Effect of ZK 62711 on levels of cyclic AMP in rat cerebral cortical slices in the presence of adenosine deaminase

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Adenosine deaminase (10 μg/ml; see ref. 13) and various concentrations of ZK 62711 were added 2 min prior to 100 μM norepinephrine. Final incubations were then carried out for 10 min. Values represent means \pm standard errors for five separate experiments.

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ase inhibitors (8, 10), ZK 62711 increased cyclic AMP levels in brain slices primarily by mechanisms not involving adenosine. In the presence of 0.5 mM theophylline, the accumulation of cyclic AMP elicited in rat cerebellar slices by 100 μ M ZK 62711 was reduced by only about 25% (results not shown), while the accumulation elicited by 100 μ M Ro 20-1724 was completely blocked. ZK 62711 enhanced the accumulation of cyclic AMP elicited by norepinephrine by about 3-fold in the presence of adenosine deaminase. The EC_{50} for ZK 62711-elicited enhancement of the response to norepinephrine was about 2 μ M, which would appear somewhat greater than its EC_{50} in the absence of adenosine deaminase. The reduction in the response to norepinephrine in the presence of adenosine deaminase presumably is due in large part to elimination of synergistic interactions of the catecholamine with endogenous adenosine.

The accumulations of cyclic AMP elicited in rat cerebral cortical slices by 1–100 μ M norepinephrine were enhanced by 1 μ M ZK 62711 (Fig. 5). The most marked increases occurred at the lowest concentrations of norepinephrine, where accumulations of cyclic AMP were quite low. The EC_{50} for norepinephrine, about 3 μ M, was,

however, not significantly altered by the presence of ZK 62711.

The effects of ZK 62711 and Ro 20-1724 on the accumulations of cyclic AMP elicited by various stimulatory agents in rat cerebral cortical slices were compared (Table 1). ZK 62711 at 1 and 100 μ M enhanced the response to norepinephrine, isoproterenol, and histamine, as did 100 μ M Ro 20-1724. Interestingly, ZK 62711 at 100 μ M did not appear to enhance the response to 100 μ M dopamine. It has been suggested that the accumulation of cyclic AMP elicited by dopamine in rat cortical slices was slightly enhanced by 250 μ M Ro 20-1724 (30). The enhancement of the response to adenosine by ZK 62711 in rat cerebral cortical slices was significant only at 100 μ M ZK 62711 (Table 1). Ro 20-1724 at 100 μ M had no effect on the adenosine response. Ro 20-1724 at higher concentrations does enhance adenosine responses (9, 11, 12).

Levels of cyclic GMP were measured concurrently in four of the experiments of Table 1. Norepinephrine and adenosine slightly increased cyclic GMP levels in rat cerebral cortical slices (Table 2). Similar responses of cyclic GMP-generating systems to norepinephrine and adenosine have been observed in guinea pig cerebral cortical slices (13). Basal levels of cyclic

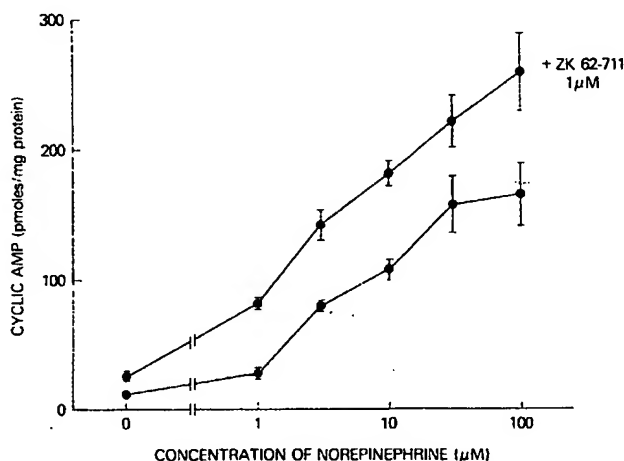


FIG. 5. Dose-response relationship for norepinephrine-stimulated accumulation of cyclic AMP in rat cerebral cortical slices in the presence and absence of ZK 62711

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). ZK 62711 (1 μ M) was added 2 min prior to norepinephrine. The final incubations with various concentrations of norepinephrine were carried out for 10 min. Values represent means \pm standard errors for four separate experiments.



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TABLE 1

Effects of ZK 62711 and Ro 20-1724 on accumulation of cyclic AMP elicited by amines and adenosine in rat cerebral cortical slices

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Either ZK 62711 or Ro 20-1724 was added 2 min prior to the amine or adenosine. The final incubations were then carried out for 10 min. Values represent the means \pm standard errors for five separate experiments.

| Additions | Cyclic AMP | | | |
|------------------------------|------------------|--------------------------|------------------|--------------------------|
| | Control | ZK 62711 | | Ro 20-1724 (100 μ M) |
| | | 1 μ M | 100 μ M | |
| | | <i>pmoles/mg protein</i> | | |
| Basal (no additions) | 12.1 \pm 1.8 | 28.0 \pm 4.5 | 68.0 \pm 6.7 | 48.9 \pm 4.0 |
| Norepinephrine (100 μ M) | 166.0 \pm 25.2 | 264.0 \pm 29.2 | 325.1 \pm 40.3 | 242.7 \pm 30.6 |
| Adenosine (100 μ M) | 188.6 \pm 22.2 | 224.0 \pm 24.8 | 297.0 \pm 27.8 | 183.0 \pm 15.0 |
| Isoproterenol (10 μ M) | 59.1 \pm 3.9 | 131.7 \pm 23.4 | 163.9 \pm 9.7 | 131.9 \pm 18.1 |
| Histamine (100 μ M) | 24.2 \pm 3.5 | 65.9 \pm 5.0 | 104.2 \pm 11.4 | 86.6 \pm 18.9 |
| Dopamine (100 μ M) | 21.8 \pm 2.4 | | 82.2 \pm 7.4 | |

TABLE 2

Effects of ZK 62711 and Ro 20-1724 on accumulation of cyclic GMP elicited by norepinephrine and adenosine in rat cerebral cortical slices

Slices from four of the experiments of Table 1 were assayed for cyclic GMP. Values are means \pm standard errors.

| Additions | Cyclic GMP | | |
|------------------------------|-----------------|--------------------------|--------------------------|
| | Control | ZK 62711 (100 μ M) | Ro 20-1724 (100 μ M) |
| | | <i>pmoles/mg protein</i> | |
| Basal (no additions) | 0.29 \pm 0.07 | 0.41 \pm 0.09 | 0.39 \pm 0.05 |
| Norepinephrine (100 μ M) | 0.61 \pm 0.12 | 0.53 \pm 0.07 | 0.53 \pm 0.08 |
| Adenosine (100 μ M) | 0.54 \pm 0.07 | 0.51 \pm 0.07 | 0.48 \pm 0.11 |

GMP in rat cerebral cortical slices after incubation for a shorter period were reported by Palmer and Duszynski (31) to be many times higher than the present results. In the presence of 100 μ M ZK 62711 or Ro 20-1724, basal levels of cyclic GMP in cortical slices tended to increase, but the increases were not statistically significant (Table 2). In the presence of the phosphodiesterase inhibitors, neither norepinephrine nor adenosine had any significant effect on cyclic GMP levels.

In rat cerebellar slices, 10 and 100 μ M ZK 62711 increased basal levels of cyclic AMP by 2- and 5-fold, respectively, over basal values. Ro 20-1724 at 10 μ M caused a 3-fold increase in basal levels. Neither compound had any effect on basal levels of cyclic GMP.

The depolarizing agent veratridine elicits significant accumulations of both cyclic AMP and cyclic GMP in cerebellar slices from mouse (23, 32) and guinea pig (13). The accumulations of cyclic AMP elicited

by veratridine in brain slices appear to involve depolarization and a resultant increase in release of adenosine (29, 33, 34). The accumulation of cyclic GMP elicited by veratridine in cerebellar slices does not appear to involve depolarization-evoked "release" of adenosine, and the mechanism remains unknown (32, 35). Because of the marked effects of veratridine on both cyclic AMP and cyclic GMP levels in cerebellar slices, it was the stimulatory agent of choice for studies of the effects of ZK 62711 and Ro 20-1724 on stimulation of both cyclic AMP- and cyclic GMP-generating systems.

ZK 62711 at 0.1–10 μ M elicited about a 2-fold increase in the accumulation of cyclic AMP produced by veratridine, while at 100 μ M ZK 62711 an even larger accumulation of cyclic AMP occurred (Table 3). The response of cyclic AMP-generating systems to a combination of 100 μ M Ro 20-1724 and veratridine was much smaller than the response to a combination of 100 μ M ZK

by amines and adenosine in rat

Methods. Either ZK 62711 or Ro 20-1724 was then carried out for 10 min. Values represent means \pm standard errors for four separate experiments.

| | Ro 20-1724 (100 μ M) |
|----------------|--------------------------|
| 100 μ M | |
| Basal | |
| 3.0 \pm 6.7 | 48.9 \pm 4.0 |
| 5.1 \pm 40.3 | 242.7 \pm 30.6 |
| 7.0 \pm 27.8 | 183.0 \pm 15.0 |
| 3.9 \pm 9.7 | 131.9 \pm 18.1 |
| 1.2 \pm 11.4 | 86.6 \pm 18.9 |
| 2.2 \pm 7.4 | |

by norepinephrine and adenosine

1P. Values are means \pm standard

| 4P | Ro 20-1724 (100 μ M) |
|-----------|--------------------------|
| 0 μ M | |
| roten | |
| 09 | 0.39 \pm 0.05 |
| 07 | 0.53 \pm 0.08 |
| 07 | 0.48 \pm 0.11 |

in brain slices appear to be a result of adenosine (29, 33, 34). The effect of cyclic GMP elicited in cerebellar slices does not involve depolarization-evoked adenosine, and the mechanism is unknown (32, 35). Because of the effects of veratridine on both cyclic AMP and cyclic GMP levels in cerebellar slices, the stimulatory agent of cyclic AMP, the effects of ZK 62711 on stimulation of both and cyclic GMP-generating

0.1–10 μ M elicited about a 2-fold increase in the accumulation of cyclic AMP by veratridine, while at 100 μ M even larger accumulation occurred (Table 3). The effect of cyclic AMP-generating systems in the presence of 100 μ M Ro 20-1724 and is much smaller than the combination of 100 μ M ZK

TABLE 3

Effects of ZK 62711 and Ro 20-1724 on accumulations of cyclic nucleotides elicited by veratridine in rat cerebellar slices

Slices were first incubated for 40 min, divided into separate portions, and incubated for an additional 60 min (see MATERIALS AND METHODS). ZK 62711 or Ro 20-1724 was added 2 min prior to veratridine. The final incubations were carried out for 10 min. Values represent means \pm standard errors for four separate experiments.

| Additions | Cyclic AMP | Cyclic GMP |
|--------------------------|-------------------|------------------|
| | pmoles/mg protein | |
| Basal (no additions) | 62.8 \pm 2.7 | 18.7 \pm 3.8 |
| ZK 62711 (0.1 μ M) | 66.6 \pm 14.2 | 14.6 \pm 3.9 |
| ZK 62711 (1 μ M) | 75.4 \pm 10.5 | 16.7 \pm 2.3 |
| ZK 62711 (10 μ M) | 128.2 \pm 13.3 | 19.5 \pm 4.6 |
| ZK 62711 (100 μ M) | 317.6 \pm 42.4 | 16.7 \pm 3.6 |
| Ro 20-1724 (100 μ M) | 192.3 \pm 42.6 | 21.9 \pm 6.7 |
| Veratridine (50 μ M) | 191.8 \pm 13.3 | 51.3 \pm 8.0 |
| ZK 62711 (0.1 μ M) | 370.2 \pm 19.3 | 66.1 \pm 14.7 |
| ZK 62711 (1 μ M) | 360.2 \pm 30.6 | 59.9 \pm 6.7 |
| ZK 62711 (10 μ M) | 373.6 \pm 38.3 | 66.3 \pm 6.7 |
| ZK 62711 (100 μ M) | 711.6 \pm 64.0 | 101.5 \pm 17.0 |
| Ro 20-1724 (100 μ M) | 458.3 \pm 78.4 | 78.1 \pm 12.1 |

62711 and veratridine, and indeed was only slightly greater than additive; i.e., no significant enhancement of the veratridine component by Ro 20-1724 was found.

Concentrations of ZK 62711 of 0.1–10 μ M had no significant effect on veratridine-elicited accumulations of cyclic GMP in rat cerebellar slices (Table 3). Only at 100 μ M did ZK 62711 significantly enhance the response to veratridine. Ro 20-1724 at 100 μ M slightly enhanced the accumulation of cyclic GMP elicited by veratridine.

The results with brain slices suggested that ZK 62711 had potent inhibitory effects on cyclic AMP phosphodiesterases, perhaps greater at low levels of cyclic AMP, and, in addition, had weaker inhibitory effects on cyclic GMP phosphodiesterases. The effects of ZK 62711 and Ro 20-1724 on soluble and particulate phosphodiesterases from rat cerebrum were therefore investigated (Table 4). The calcium-dependent activator protein and calcium ions were added to ensure that the major phosphodiesterase activity measured in the preparations would be the calcium-dependent phosphodiesterase (see refs. 2, 27, 36, 37). In both rat cerebral cortex and rat cerebellum the major phosphodiesterase isozyme

is calcium-dependent (2, 16, 38, 39).

Both ZK 62711 and Ro 20-1724 at 1 μ M inhibited the hydrolysis of cyclic AMP by soluble and particulate phosphodiesterases from rat cerebrum, while having virtually no effect on the hydrolysis of cyclic GMP (Table 4). At 100 μ M both ZK 62711 and Ro 20-1724 inhibited the hydrolysis of cyclic AMP by soluble and particulate brain phosphodiesterases by about 50%, and inhibition of cyclic GMP hydrolysis was significant. Ro 20-1724 has been reported to inhibit the hydrolysis of cyclic AMP but not cyclic GMP in homogenates from neuroblastoma cells and brain tissue (40, 41).

In view of the apparent somewhat similar potency of ZK 62711 and Ro 20-1724 toward the inhibition of phosphodiesterases in cell-free preparations (Table 4) and the nearly 100-fold greater potency of

TABLE 4

Effects of ZK 62711 and Ro 20-1724 on hydrolysis of cyclic AMP and cyclic GMP by phosphodiesterases of soluble and particulate preparations of rat brain homogenates

Phosphodiesterase activities were assayed at 1 μ M substrate concentration in the presence of 1 μ g of activator protein and 0.04 mM calcium ions (see MATERIALS AND METHODS). The control activities of the cyclic AMP phosphodiesterase were 2.0 nmoles/mg of protein per minute for the soluble preparation and 1.5 nmoles/mg of protein per minute for the particulate preparation. The control activities of the cyclic GMP phosphodiesterase were 8.9 nmoles/mg of protein per minute for the soluble preparation and 3.2 nmoles/mg of protein per minute for the particulate preparation. The enzyme activities are expressed as percentage inhibition relative to the control. Each value is the mean of triplicate determinations.

| Additions | Inhibition | |
|--------------------------|------------|------------|
| | Cyclic AMP | Cyclic GMP |
| | % | % |
| Soluble preparation | | |
| ZK 62711 (1 μ M) | 35.8 | 5.0 |
| ZK 62711 (100 μ M) | 56.0 | 30.7 |
| Ro 20-1724 (1 μ M) | 12.0 | 5.9 |
| Ro 20-1724 (100 μ M) | 50.0 | 23.4 |
| Particulate preparation | | |
| ZK 62711 (1 μ M) | 23.1 | 5.5 |
| ZK 62711 (100 μ M) | 55.0 | 28.4 |
| Ro 20-1724 (1 μ M) | 32.8 | 5.9 |
| Ro 20-1724 (100 μ M) | 56.0 | 45.3 |

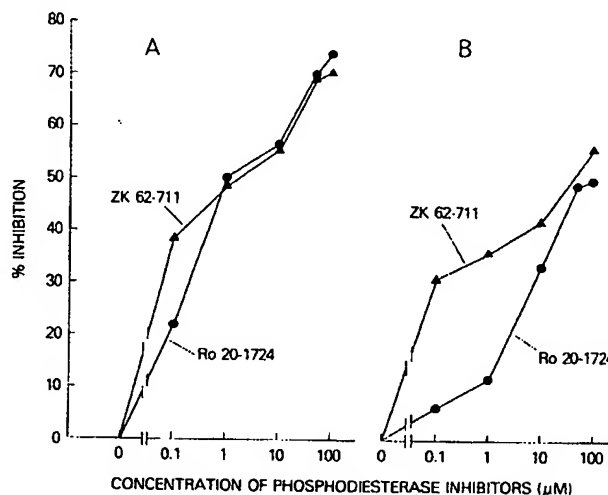


FIG. 6. Effects of ZK 62711 and Ro 230-1724 on hydrolysis of cyclic AMP by phosphodiesterases from rat cerebrum in the presence of EGTA (A) or calcium ions and calcium-dependent activator protein (B)

Phosphodiesterase activities of the soluble fraction from rat cerebrum (see MATERIALS AND METHODS) were assayed at 1 μ M cyclic AMP in the presence of either 2 mM EGTA (A) or 1 μ g of activator protein and 40 μ M calcium ions (B). The control activity with EGTA was 1.27 nmoles/mg of protein per minute, while the control activity in the presence of activator and calcium ions was 2.01 nmoles/mg of protein per minute. The enzyme activities are expressed as percentage inhibition relative to the control. Each value is the mean of triplicate determinations.

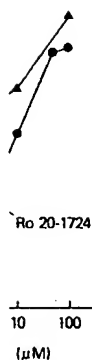
ZK 62711 toward the potentiation of accumulations of cyclic AMP in rat cortical slices (Fig. 3), further experiments on inhibition of phosphodiesterases by the two compounds were performed (Fig. 6). With soluble rat cerebral phosphodiesterase, in the absence of added calcium-dependent activator protein, and with EGTA added, ZK 62711 appeared only marginally more potent than Ro 20-1724 as a phosphodiesterase inhibitor (Fig. 6A). However, in the presence of excess calcium-dependent activator protein and calcium ions, dose-response relationships revealed that ZK 62711 at low concentrations was nearly 100 times more potent than Ro 20-1724 as a phosphodiesterase inhibitor (Fig. 6B). Neither Ro 20-1724 nor ZK 62711 has structural features necessary for chelation of calcium ions.

DISCUSSION

The phenyl-2-pyrrolidone ZK 62711 is closely related in structure to the phosphodiesterase inhibitor Ro 20-1724 (Fig. 1). Both compounds have central depressant

activity, and, as shown in the present paper, in brain preparations ZK 62711 shares the phosphodiesterase inhibitor activity of Ro 20-1724. In brain slices ZK 62711, based on marked enhancement of amine- and adenosine-elicited accumulations of cyclic AMP, appeared to be a potent phosphodiesterase inhibitor (Fig. 2 and Table 1). Indeed, ZK 62711 was nearly 100 times more potent than Ro 20-1724 in enhancing the response of cyclic AMP-generating systems to norepinephrine in rat cerebral cortical slices (Fig. 3). Unlike Ro 20-1724 (13), an enhanced adenosine component of cyclase activation appeared to be relatively unimportant to the effects of ZK 62711 on cyclic AMP levels, since ZK 62711 markedly increased basal and norepinephrine-stimulated levels of cyclic AMP even in the presence of theophylline or adenosine deaminase (Fig. 4). ZK 62711 appeared more potent in enhancing accumulations of cyclic AMP elicited by amines such as norepinephrine, isoproterenol, and histamine than in enhancing adenosine-elicited accumulations of cyclic AMP (Fig. 2 and Table 1).

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MATERIALS AND METHODS) were of activator protein and 40 μ M protein per minute, while the /mg of protein per minute. The rol. Each value is the mean of

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In rat cerebral cortical slices, neither ZK 62711 nor Ro 20-1724 had a significant effect on basal levels of cyclic GMP, and actually reduced the norepinephrine- and adenosine-elicited accumulations of cyclic GMP (Table 2). Clearly, both compounds appeared to have selective effects on cyclic AMP- rather than cyclic GMP-generating systems.

In rat cerebellar slices both compounds increased basal levels of cyclic AMP, but neither compound had any effect on basal levels of cyclic GMP (Table 3). Somewhat higher concentrations of ZK 62711 appeared to be required to elevate basal levels of cyclic AMP in cerebellar slices as compared to cerebral cortical slices. Perhaps this is a reflection of the already high basal levels of cyclic AMP in cerebellar slices.

The responses of cyclic AMP-generating systems in rat cerebellar slices to veratridine were markedly potentiated by ZK 62711 at concentrations of 0.1–100 μ M (Table 3). The accumulations of cyclic AMP elicited in brain slices by this depolarizing agent have been shown to involve, at least in part, depolarization-elicited release of adenosine (8, 29, 33, 34), but release of other factors or direct effects of depolarization cannot be discounted. Clearly, however, ZK 62711, even at 100 μ M, does not have sufficient local anesthetic activity to decrease the requisite depolarizing action of veratridine. Local anesthetics can completely block the response to veratridine (34). The response of cyclic AMP-generating systems to combinations of veratridine and 100 μ M Ro 20-1724 were not significantly greater than additive, while the responses to combinations of veratridine and ZK 62711 were in all cases greater than additive. At 100 μ M, both ZK 62711 and, to a lesser extent, Ro 20-1724 enhanced veratridine-elicited accumulations of cyclic GMP in rat cerebellar slices. Clearly, in cerebellum ZK 62711 was relatively impotent with regard to enhancement of responses of cyclic GMP-generating systems.

The results with brain slices provided indirect evidence that ZK 62711 was a potent inhibitor of phosphodiesterases associated with amine- and adenosine-respon-

sive cyclic AMP-generating systems in intact cells of brain slices. Confirmation of this conclusion required an investigation of the effect of ZK 62711 on brain phosphodiesterases in cell-free preparations. The initial investigation of ZK 62711 with phosphodiesterases revealed it to be an inhibitor that resembled Ro 20-1724 in its potency and selectivity toward cyclic AMP phosphodiesterases rather than cyclic GMP phosphodiesterases (Table 4; cf. refs. 41, 40). Soluble phosphodiesterase from rat cerebrum did appear to be inhibited somewhat more effectively by low concentrations of ZK 62711 than by Ro 20-1724, but this was not true of the particulate enzyme. Dose-response relationships for inhibition of phosphodiesterases from rat brain by ZK 62711 and Ro 20-1724 revealed that in the presence of EGTA ZK 62711 was only marginally more potent than Ro 20-1724 (Fig. 6A). However, in the presence of added calcium-dependent activator protein and calcium ions, ZK 62711 at low concentrations was nearly 100 times more potent as a phosphodiesterase inhibitor than Ro 20-1724 (Fig. 6B). The 100-fold difference in potencies of the two compounds as inhibitors of calcium-dependent cyclic AMP phosphodiesterase mirrors the 100-fold difference in potencies toward enhancement of norepinephrine-stimulated accumulation of cyclic AMP in brain slices. The parallelism between the potencies of ZK 62711 with cell-free, calcium-dependent phosphodiesterases and with intact cell preparations strongly suggests that the potentiation of amine responses of cyclic AMP-generating systems by ZK 62711 is due to inhibition of a calcium-dependent phosphodiesterase associated with amine-responsive cyclases. It is also tempting to speculate that the apparent lower potency of ZK 62711 in brain slices in the presence of adenosine is due to the involvement of another phosphodiesterase with adenosine-activated systems. Further studies with ZK 62711 on high- and low- K_m phosphodiesterase activity and on isozymes from brain are clearly needed. The present data, however, reveal ZK 62711 to be the first member of a new class of phosphodiesterase inhibitors related in

structure to Ro 20-1724, but, at least with respect to calcium-dependent cyclic AMP phosphodiesterase activity from brain, much more potent. The similar high potencies of ZK 62711 with both cell-free and intact cell systems from brain and its lack of apparent effects on "release" of adenosine or inhibition of responses to adenosine suggest it to be the agent of choice for study of the role of phosphodiesterases in control of accumulations of cyclic AMP in the central nervous system.

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Structure-Activity Relationships for Inhibitors of Phosphodiesterase from Erythrocytes and Other Tissues

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I. INTRODUCTION

Several years ago we decided to enter the world of cyclic AMP by studying the adenylyl cyclase of a relatively simple cell, preferably one with a single membrane. This point of attack seemed most logical because of the high order of specificity found for the activation of the adenylyl cyclases of various tissues. While the regulation of adenosine-3',5'-cyclic phosphate, adenosine-5'-phosphate-3'-phosphohydrolase, EC 3.1.4.c (phosphodiesterase) could also regulate intracellular cyclic AMP levels, the ubiquitous distribution and apparent homogeneity of this enzyme did not seem to provide the basis for a tissue-specific attack on the cyclic AMP systems. In any event, during our studies with adenylyl cyclase, we did not ignore phosphodiesterase. Our search for a simple adenylyl cyclase led us to study the non-nucleated erythrocytes of several mammalian species. We confirmed its absence in the dog erythrocyte but found, as we had hoped, that the erythrocyte of the rat and mouse had a cyclase (Sheppard and Burghardt, 1969) and that the enzyme of the rat was activated only by catecholamines and prostaglandin (Sheppard and Burghardt, 1970). An active phosphodiesterase was also present (Sheppard and Burghardt, 1970) but initially our attention was focused only on the adenylyl cyclase of the ghosts of the rat erythrocyte.

At about this time, investigations were being carried out on an interesting drug, Ro 7-2956, which lowered the blood pressure of animals and markedly stimulated the heart. These cardiac effects could be maintained in the presence of both α - and β -blocking agents (Pettinger, Osborne, and Moe, 1969). Ro 7-2956 was shown to be lipolytic with isolated fat cells even in the presence of

the β -blocking agent propranolol and to be able to potentiate the lipolytic action of cyclic AMP (Dalton, Quinn, Burghardt, and Sheppard, 1970). Since β -agonists appear to activate adenylyl cyclase (Robison, Butcher, and Sutherland, 1969), it was reasoned that this drug was most probably acting beyond the adenylyl cyclase system, perhaps at the level of phosphodiesterase. It was tested on the phosphodiesterase from the fat cell of the rat and found to be more potent than theophylline.

II. INHIBITION BY ANALOGUES OF RO 7-2956

Several analogues of this compound were prepared, and a very interesting structure-activity relationship emerged when these analogues were tested for their ability to inhibit rat erythrocyte phosphodiesterase (Sheppard and Wiggan, 1971a). The compounds shown in Table 1 belong to a family of 4-(3,4-dialkoxybenzyl)-2-imidazolidinones. The compound Ro 7-2956 contains CH_3 groups on the oxygens in the 3 and 4 positions of the benzyl moiety and has an I_{50} of 12.0 μM . Increasing the size of the alkyl group attached to the oxygen at the 3 position increased the potency markedly such that butyl > isopropyl > ethyl > methyl analogue. Lengthening the side chain on the 4-oxygen had the opposite effect; it lowered the potency. The introduction of an alcoholic group on the ethoxy side chain of the 3-oxygen also lowered the activity (compound XII vs. XIV). The substitution of hydrogens in place of the methyl groups

TABLE 1. Inhibition of rat erythrocyte phosphodiesterase by Ro 7-2956 and its analogues



| Compound | R ₁ | R ₂ | I ₅₀ (μM) |
|------------|---------------------------------|---------------------------------|-----------------------------------|
| Ro 7-2956 | CH_3 | CH_3 | 12.0 |
| XIV | C_2H_5 | CH_3 | 0.7 |
| XII | $\text{C}_2\text{H}_5\text{OH}$ | CH_3 | 9.0 |
| XV | $\text{CH}(\text{CH}_3)_2$ | CH_3 | 0.5 |
| Ro 20-1724 | C_4H_9 | CH_3 | 0.1 |
| Ro 20-0404 | CH_3 | C_2H_5 | 32.0 |
| XIII | C_2H_5 | C_2H_5 | 2.4 |
| XI | C_2H_5 | $\text{C}_2\text{H}_5\text{OH}$ | 24.0 |
| VI | H | H | Inactive |
| IX | CH_3 | H | 54.0 |

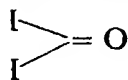
The phosphodiesterase activity and its inhibition were measured by incubating with cyclic AMP- ^3H according to the procedure described previously (Dalton et al., 1970; Sheppard and Wiggan, 1971a).

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| R_2 | I_{50} (μM) |
|-----------------------|----------------------------|
| C_3 | 12.0 |
| C_3 | 0.7 |
| C_3 | 9.0 |
| C_3 | 0.5 |
| C_3 | 0.1 |
| H_5 | 32.0 |
| H_3 | 2.4 |
| H_3OH | 24.0 |
| | Inactive |
| | 54.0 |

measured by incubating with cyclic
(Dalton et al., 1970; Sheppard and

resulted in a completely inactive compound. Methylation of the 3-hydroxyl group yielded an active compound which was almost 10 times more potent than theophylline. Methylation of the 4-hydroxy group, of course, yielded Ro 7-2956 and a fourfold increase in potency. The 3-butoxy-4-methoxy derivative (Ro 20-1724) was the most potent compound found to date with an I_{50} of about $0.1 \mu\text{M}$.

From these studies it was suggested that a hydrophobic cavity existed or was readily produced on the surface of the enzyme with a high degree of acceptance for the alkoxy group. If the larger or more hydrophobic group was in the 3-position, the remainder of the molecule was positioned for good inhibition. With the large hydrophobic group attached to the oxygen in the 4-position, the remainder of the molecule was apparently positioned poorly in the vicinity of the inhibitory site. While the imidazolidinone group has been shown to be important for inhibition, its configuration around position 4 was not too critical. The stereoisomers of Ro 7-2956 were both quite active, with the *l*-isomer being more potent by a factor of three. With Ro 20-1724, however, the two isomers were equipotent.

Kinetic analyses of the phosphodiesterase revealed a K_m for cyclic AMP of 10^{-5} M and an inhibition by Ro 20-1724 and theophylline of a mixed type, with both competitive and noncompetitive aspects (Sheppard and Wiggan, 1971a).

III. INHIBITION OF PHOSPHODIESTERASE FROM SEVERAL TISSUES

Concurrent with the evaluation of these compounds (Table 2) as inhibitors of phosphodiesterase was an examination of their antihypertensive activity by our Cardiovascular Research Section headed by Dr. William Pettinger. There existed a high coefficient of correlation between these two activities, but no such correlation could be found between cardiac stimulation and enzyme inhibition. It was therefore believed that the properties of the enzyme from the erythrocyte differed from that of the heart and other tissues. The phosphodiesterase activity of 1.15% KCl homogenates of a variety of tissues of the dog were then examined to determine their sensitivities to several inhibitors.

In the second column of Table 2 it is shown that of the canine tissues studied, only the erythrocyte enzyme was sensitive to $0.1 \mu\text{M}$ Ro 20-1724, suggesting that the type of tissue may be of greater importance than the animal species in determining the nature of the phosphodiesterase. The order of decreasing potency as inhibitors of the dog erythrocyte enzyme was Ro 20-1724, papavarine, theophylline, and 3-methoxytyramine. With the mesenteric artery, heart, fat, cerebral cortex, and caudate nucleus, the order was the same, with the glaring exception of Ro 20-1724, which was inactive at 10^{-7} M. The

TABLE 2. Inhibition of phosphodiesterase from several tissues of the dog by theophylline, Ro 20-1724, 3-methoxy-tyramine, and papavarine

| Canine tissue | % Inhibition of phosphodiesterase activity | | | |
|-------------------|--|-----------------------------|-------------------------------|---------------------------|
| | Theophylline (0.5 mM) | Ro 20-1724 (0.1 μ M) | 3-Methoxy- tyramine (5 mM) | Papavarine (7 μ M) |
| erythrocyte | 37.5 | 32.0 | 57.5 | 26.5 |
| mesenteric artery | 44.3 | 0.25 | 54.3 | 41.6 |
| heart | 47.1 | 1.5 | 57.5 | 73.7 |
| fat | 44.8 | 1.1 | 35.7 | 41.8 |
| cerebral cortex | 38.2 | 0.75 | 46.8 | 22.4 |
| caudate nucleus | 40.9 | 0.25 | 34.3 | 39.6 |

The phosphodiesterase preparation from the erythrocytes was a 1:12 hemolysate, while that from other tissues was the 105,000 \times g supernatant of a 1:10 homogenate prepared with 1.15% KCl and stored frozen overnight prior to centrifugation. The results shown represent the mean of two experiments run in quadruplicate.

enzyme preparations from most of the tissues responded to about the same extent to the particular concentration of the other inhibitors used. An exception to this general picture was the greater sensitivity of heart ventricular phosphodiesterase to papavarine. Methoxytyramine was tested because it resembled the open-ring analogue of Ro 7-2956, the α -aminomethyl derivative of dimethoxyphenethylamine, which was moderately potent as an inhibitor of rat erythrocyte phosphodiesterase, with an I_{50} of 420 μ M (Sheppard et al., 1971a).

The finding that the mesenteric artery phosphodiesterase lacked the sensitivity of the erythrocyte enzyme to Ro 20-1724 was disappointing and seemed to mediate against any correlation existing between inhibition of the mesenteric artery enzyme and antihypertensive activity. Any such correlation, however, would require that the potencies of several inhibitors be in the proper order when tested against the enzymes from tissues likely to regulate blood pressure. The three substances tested in Table 3 are all imidazolidinone derivatives, with the antihypertensive activity of Ro 20-1724 > Ro 7-2956 > Ro 20-0404. This order of potency was observed with the phosphodiesterase of the renal artery and portal vein, although the differences are not very great. Estimates of the I_{50} for Ro 20-1724 and Ro 20-0404 were 280 μ M and 870 μ M respectively with the renal artery phosphodiesterase, as compared with 0.1 μ M and 32 μ M with the erythrocyte enzyme. The I_{50} values with a rat skeletal muscle enzyme preparation were 20 μ M and 210 μ M for Ro 20-1724 and Ro 20-0404 respectively. Thus all of these tissues have phosphodiesterases of the type which was much less sensitive than the rat erythrocyte enzyme to this class of inhibitors. The rat skeletal muscle enzyme appears to be of an intermediate type. One must consider the possibility that the erythrocyte type of enzyme was only a small fraction of the total phosphodiesterase activity but

tissues of the dog by theophylline, and papavarine

phosphodiesterase activity

| 3-Methoxy-tyramine (5 mM) | Papavarine (7 μ M) |
|---------------------------|------------------------|
| 57.5 | 26.5 |
| 54.3 | 41.6 |
| 57.5 | 73.7 |
| 35.7 | 41.8 |
| 46.8 | 22.4 |
| 34.3 | 39.6 |

tes was a 1:12 hemolysate, while that 10 homogenate prepared with 1.15% e results shown represent the mean of

responded to about the same r inhibitors used. An exception of heart ventricular phospho- is tested because it resembled minomethyl derivative of di- / potent as an inhibitor of rat 0 μ M (Sheppard et al., 1971 a). phosphodiesterase lacked the -1724 was disappointing and ting between inhibition of the activity. Any such correlation, eral inhibitors be in the proper ssues likely to regulate blood le 3 are all imidazolidinone f Ro 20-1724 > Ro 7-2956 > with the phosphodiesterase of differences are not very great. 404 were 280 μ M and 870 μ M ase, as compared with 0.1 μ M I_{50} values with a rat skeletal 0 μ M for Ro 20-1724 and Ro ave phosphodiesterases of the it erythrocyte enzyme to this me appears to be of an inter- that the erythrocyte type of hosphodiesterase activity but

TABLE 3. Effect of inhibitors on the phosphodiesterase activity of vascular tissue

| Tissue | % Inhibition at 5×10^{-4} M | | |
|----------------------------|--------------------------------------|-----------|------------|
| | Ro 20-1724 | Ro 7-2956 | Ro 20-0404 |
| renal artery | 60.4 | 49.3 | 40.8 |
| femoral artery | 54.5 | 37.0 | 37.1 |
| mesenteric artery | 39.5 | 30.3 | 34.6 |
| superior mesenteric artery | 34.5 | 21.5 | 20.8 |
| pulmonary artery | 16.0 | 19.5 | 17.5 |
| portal vein | 53.4 | 44.3 | 38.2 |
| vena cava | 28.6 | 10.7 | 14.6 |

The phosphodiesterase from the various tissues was prepared as described in Table 2. The results shown represent the mean of quadruplicate analyses. The three inhibitors were run at the same time with each tissue under study.

that *in situ* it was confined to an essential component of the vascular tissue, probably the smooth muscle cell. This possibility needs to be examined in greater detail.

It was of some interest to note that the sensitivities of the phosphodiesterases from vascular tissue to Ro 20-1724 differed to some extent, with the enzyme of the renal artery being the most and the pulmonary artery the least sensitive to all three inhibitors.

It was now clear that there was something very different about the phosphodiesterase of erythrocytes as compared to that of other tissues. The reasons for this are unknown, but it is suggested that they are related to differences in the structure and composition of the enzymes.

IV. PHOSPHODIESTERASE INHIBITION AND THE DOPAMINE RECEPTOR

The activity demonstrated earlier with the metabolite of dopamine, 3-methoxytyramine, prompted us to examine the various catecholamines and their metabolites as inhibitors of phosphodiesterase of the dog cerebral cortex and erythrocyte. In Table 4 it can be seen that dopamine, norepinephrine, and their metabolites are relatively inactive with the erythrocyte phosphodiesterase. Inhibition of the enzyme from the cerebral cortex, however, was obtained with dopamine, 3,4-dihydroxyphenylacetic acid, and 4-hydroxy-3-methoxy phenylacetic acid, but not with their β -hydroxylated derivatives. This now provided a system at a molecular level which demonstrated an increased potency of dopamine and its derivatives over that of norepinephrine. In addition, it further differentiated the erythrocyte and cerebral cortex phosphodiesterase. One is forced to ask if this activity could in any way be associated

TABLE 4. Inhibition of phosphodiesterase from the erythrocyte and cerebral cortex of the dog

| Compound | % Inhibition at 10^{-5} M | | |
|---------------------------------------|-----------------------------|-----------------|--------|
| | Erythrocyte | Cerebral Cortex | |
| | | Exp. 1 | Exp. 2 |
| dopamine | 10.7 | 43.4 | — |
| norepinephrine | 12.7 | 7.1 | — |
| 3-methoxy-tyramine | — | — | 22.1 |
| normetanephrine | — | — | 2.5 |
| 3,4-dihydroxy-phenylacetic acid | 8.2 | 72.4 | 48.0 |
| 3,4-dihydroxy-mandelic acid | 9.0 | 9.1 | — |
| 4-hydroxy-3-methoxy-phenylacetic acid | 5.2 | 20.7 | — |
| 4-hydroxy-3-methoxy-mandelic acid | 3.0 | 6.2 | — |

Each value represents the mean of quadruplicate analyses.

with what is described pharmacologically as specific dopamine receptors.

According to Ernst (1969) and Goldberg, Sonnevile, and McNay (1968), dopamine action on its specific receptors was expressible even in the presence of α - and β -blockade. Norepinephrine was only weakly active if at all. As mentioned previously, the imidazolidinone group of compounds, like dopamine, caused vasodilation even in the presence of α - and β -blockade. It would be expected that inhibitors of phosphodiesterase would be poorly affected by α - and β -blockade except at very high doses. In support of the idea that inhibition of phosphodiesterase may be a component of the action of dopamine at its receptor was the finding that apomorphine inhibited the cerebral cortex enzyme with an I_{50} of $15 \mu\text{M}$ (Sheppard and Wiggan, 1971b). Thus apomorphine was 40 times more potent than theophylline and about equipotent with papavarine. Apomorphine has been reported by Ernst (1969) and Goldberg et al., (1968) to act on dopamine receptors. These supportive observations must be balanced by the findings that dopamine and its metabolites were weak inhibitors of phosphodiesterase from dog cerebral cortex. The inhibition was no greater in preparations from the caudate nucleus which possesses specific dopamine receptors. This hypothesis obviously needs more rigorous testing.

V. PROPERTIES OF PHOSPHODIESTERASE FROM CANINE CEREBRAL CORTEX

It was apparent, now, that our studies had to follow the path of enzyme purification. The validity of some of the ideas discussed above was linked to the concept that different forms of the enzyme existed and that the response obtained with any crude preparation represented the resultant of their individual activities. Following reports by Thompson and Appleman (1970)

erythrocyte and cerebral cortex of the dog

% Inhibition at 10^{-3} M

| Cerebral Cortex | |
|-----------------|--------|
| Exp. 1 | Exp. 2 |
| 43.4 | — |
| 7.1 | — |
| — | 22.1 |
| — | 2.5 |
| 72.4 | 48.0 |
| 9.1 | — |
| 20.7 | — |
| 6.2 | — |

ses.

specific dopamine receptors. Sonnevile, and McNay (1968), expressible even in the presence weakly active if at all. As men- of compounds, like dopamine, and β -blockade. It would be would be poorly affected by support of the idea that inhibi- of the action of dopamine at its inhibited the cerebral cortex (Wiggin, 1971b). Thus apo- phylline and about equipotent d by Ernst (1969) and Goldberg : supportive observations must ad its metabolites were weak ral cortex. The inhibition was cleus which possesses specific needs more rigorous testing.

PHOSPHODIESTERASE IN THE CORTICAL

to follow the path of enzyme discussed above was linked to the listed and that the response ted the resultant of their in- pson and Appleman (1970)

and by Rosen (1970) that phosphodiesterase activity was associated with different molecular weight fractions, we sought to fractionate the phosphodiesterase activity in a $105,000 \times g$ supernatant of a homogenized dog cerebral cortex by Sephadex G-200 chromatography. Three peaks of activity were noted, and some of their properties can be seen in Table 5. There are obviously

TABLE 5. Properties of phosphodiesterase in Sephadex G-1-200 fractions of a $105,000 \times g$ supernatant of dog cerebral cortex homogenate

| Fraction | Apparent* mol. wt. | K_m (10^{-5} M) | V_{max} μ moles/30 min/mg protein | Q_{10} |
|----------|-----------------------|-------------------------|---|----------|
| A | 400,000-480,000 | 0.87 | 0.13 | 1.40 |
| | | 50.0 | 3.8 | |
| B | 115,000 | 1.2 | 0.35 | 1.40 |
| | | 7.1 | 1.5 | |
| C | 48,000 | 1.54 | 0.18 | 1.78 |

*Based on columns calibrated with the globular proteins aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A.

quite large differences in apparent molecular weights, ranging from over 400,000 for fraction A to 48,000 for fraction C. These values are based on columns standardized with globular proteins, and, if the phosphodiesterases are nonglobular or conjugated proteins, the molecular weights are likely to be quite different. Lineweaver-Burke plots demonstrate that all three forms have a low K_m for cyclic AMP of the order of 10μ M. Fractions A and B have higher K_m values of 500 and 71μ M, respectively, which may be indicative of the presence of two separate enzymes. If this is true, the K_m values obtained from the Lineweaver-Burke plot may be more apparent than real. This complication was clearly described by Thompson and Appleman (1971) and emphasizes the care that must be taken in interpreting such data. Further purification of these enzymes is necessary before accurate K_m values can be obtained.

As found for the heart phosphodiesterase by O'Dea, Haddox, and Goldberg (1970), the activity of the brain enzymes was relatively insensitive to cold. The Q_{10} values listed were calculated from the difference in hydrolytic activity between 1 and 37°C and should be treated only as crude estimates. The Q_{10} for both fractions A and B was 1.40, while that of fraction C was 1.78. The Q_{10} for the rat erythrocyte phosphodiesterase was found to be 1.65.

Since fraction C was most like the erythrocyte phosphodiesterase in its K_m and Q_{10} values, an assessment was made of its sensitivity to two imidazolidinone inhibitors. The I_{50} for Ro 20-1724 was approximately 25μ M, which was about 10 times lower than that observed for the $105,000 \times g$ supernatant of the cerebral cortex prior to chromatography; it was, however, still 400 times

weaker than that reported for the erythrocyte phosphodiesterase. Ro 20-0404 was about one-tenth as potent as Ro 20-1724 as an inhibitor of fraction C phosphodiesterase, but this difference was much smaller than the 300-fold decrease seen with the erythrocyte enzyme. Examination of the other fractions confirmed the impression that fraction C was most sensitive to Ro 20-1724. It is of some interest that the sensitivity of fraction C to these inhibitors resembled that of rat skeletal muscle.

A comparison of the ability to hydrolyze cyclic AMP and cyclic GMP was made using a substrate concentration of 5×10^{-6} M. It was found that fractions A and B hydrolyzed cyclic GMP more extensively than cyclic AMP by factors of approximately 1.76 and 2.92, respectively; fraction C preferred cyclic AMP by a factor of about 1.7. The erythrocyte enzyme had a relatively small capacity to hydrolyze cyclic GMP.

The effects of inhibition with Ro 20-1724 and activation with snake venom (Cheung, 1969) on the hydrolysis of cyclic AMP and cyclic GMP are shown in Table 6. It is apparent that the hydrolysis of cyclic GMP by the phosphodiesterase from erythrocytes and cortex fractions B and C is inhibited little if at all by Ro 20-1724, which conforms with the findings of Goldberg, Lust, O'Dea, Wei, and O'Toole (1970) using rat brain phosphodiesterase. Snake venom, as reported by Cheung (1969) for rat brain phosphodiesterase, activated the cyclic AMP hydrolytic action of all three preparations, with that of fraction B being stimulated most. The hydrolysis of cyclic GMP by cortex fractions B and C was also stimulated by snake venom, while that of the erythrocyte was inhibited.

TABLE 6. Effect of Ro 20-1724 and snake venom on the hydrolysis of cyclic AMP and cyclic GMP by phosphodiesterases of erythrocyte and cortex fractions B and C

| Enzyme | Addition | % Change from control hydrolysis | |
|-------------------|---------------------------------------|----------------------------------|------------|
| | | Cyclic AMP | Cyclic GMP |
| erythrocyte | Ro 20-1724 (5×10^{-7} M) | -67.9 | -17.4 |
| | snake venom* | +44.5 | -74.3 |
| cortex fraction B | Ro 20-1724 (5×10^{-4} M) | -26.0 | 0 |
| | snake venom | +313.0 | +245.0 |
| cortex fraction C | Ro 20-1724 (5×10^{-4} M) | -86.9 | -11.0 |
| | snake venom | +134.0 | +245.0 |

* Snake venom was *Crotalus atrox* obtained from Sigma Chemical Co., and each incubation (0.25 ml) contained 10 μ g.

phosphodiesterase. Ro 20-0404 is as an inhibitor of fraction C much smaller than the 300-fold inhibition of the other fractions most sensitive to Ro 20-1724. Fraction C to these inhibitors resembles

cyclic AMP and cyclic GMP 5×10^{-6} M. It was found that more extensively than cyclic 2.92, respectively; fraction C. The erythrocyte enzyme had a K_m of 10 μ M.

and activation with snake venom phosphodiesterase and cyclic GMP are shown in Figure 1. Fraction C is inhibited little if at all by the phosphodiesterase of Goldberg, Lust, O'Dea, and Sheppard. Snake venom, as a phosphodiesterase, activated the cyclic AMP, with that of fraction B being the most active. Fraction C is inhibited little by cortex fractions B and C. The erythrocyte was inhibited.

Hydrolysis of cyclic AMP and cyclic GMP by cortex fractions B and C

| Change from control hydrolysis | |
|--------------------------------|------------|
| Cyclic AMP | Cyclic GMP |
| -67.9 | -17.4 |
| +44.5 | -74.3 |
| -26.0 | 0 |
| +313.0 | +245.0 |
| -86.9 | -11.0 |
| +134.0 | +245.0 |

Ro 20-1724, 10 μ M, and each incubation

The cyclic AMP and cyclic GMP phosphodiesterase activities could be superimposed in cortex fractions from Sephadex G-200 columns.

It is of interest that cyclic AMP-dependent protein kinase activity was present in phosphodiesterase peaks obtained by Sephadex G-200 chromatography of hemolysates and cerebral cortex extracts. It might be anticipated, therefore, that the kinetics of these phosphodiesterase preparations could be influenced by the presence of very low K_m cyclic AMP-binding proteins which possess the ability of protecting cyclic AMP from hydrolysis (O'Dea, Haddox, and Goldberg, 1971).

VI. DISCUSSION

These studies demonstrate that Ro 20-1724 is a uniquely potent inhibitor of the phosphodiesterase from the erythrocyte in contrast to that from other tissues. This has provided us with a tool for distinguishing various forms of phosphodiesterase. Ro 20-1724 also selectively inhibits the hydrolysis of cyclic AMP as compared to cyclic GMP. In our laboratories, papavarine and theophylline inhibit the hydrolysis of both cyclic nucleotides to about the same extent, confirming the findings of Goldberg et al. (1970).

In an effort to understand the unique sensitivity of the erythrocyte enzyme to Ro 20-1724, we attempted to purify the enzyme by Sephadex G-200 chromatography. Inconsistent chromatographic patterns were obtained, with major peaks of activity having apparent molecular weights from 200,000 to 400,000. It was noted that freezing followed by immediate thawing resulted in little if any change in activity, while storage in frozen state for 1 to 5 days increased the activity 50 to 100%. In both cases, an apparent conversion to a smaller molecular weight fraction was observed. This effect of freezing has not been noted with preparations from other tissues. We hope to be able to understand and control these processes so that we can develop a better picture of the actual state of phosphodiesterase in the intact cell.

A few comments about the rat erythrocyte seem appropriate here. This easily obtainable cell possesses a number of enzymes in search of a function. It possesses the elements of the cyclic AMP system which include adenylyl cyclase, phosphodiesterase, protein kinase, and cAMP binding activity. These factors are certainly not related to the turning on or turning off of a genome, since the cell possesses no nuclear or even mitochondrial DNA. The cyclic AMP system is apparently not required for the transport of oxygen, since the human erythrocyte functions well in this regard despite deficiencies in adenylyl cyclase and phosphodiesterase (Sheppard and Burghardt, 1969, 1970). Does the cyclic AMP regulate some intracellular enzymes such as phosphofructokinase, or does the system simply represent a vestige of a more glorious past? The combined efforts of several laboratories should soon provide the answer.

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